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Comparison of HCV RNA Assays for the Detection and Quantification of Hepatitis C Virus RNA Levels in Serum of Patients With Chronic Hepatitis C Treated With Interferon

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Detection and quantification of hepatitis C virus (HCV) RNA levels by using the standardized qualitative Amplicor™ HCV and quantitative Amplicor HCV Monitor™ assays (Roche Molecular Systems) were evaluated in 48 patients with chronic hepatitis C treated with interferon. Results were compared with an in-house reverse transcription and polymerase chain reaction (RT-PCR) assay and the branched DNA (bDNA) assay (Quantiplex™, version 1.0, Chiron Diagnostics). Concordance of the qualitative results with the Amplicor™ HCV and in-house RT-PCR assays occurred in 82% of the samples. All but one of the discrepant specimens were found positive by the Amplicor™ HCV assay and negative by the in-house RT-PCR. Among the samples with HCV RNA levels measurable with the Amplicor HCV Monitor™ assay, 22% had HCV RNA titers below the detection limit of the Quantiplex™ assay. A statistically significant correlation was found between the 2 quantitative assays, although lower titers were obtained with the Amplicor HCV Monitor™ assay. More important, a good correlation was observed in the evolution of viremia as measured by the 2 assays during interferon therapy. During follow-up of interferon treatments, with the Amplicor HCV Monitor™ assay, persisting viremia was still detected in 27% of the patients who normalised alanine aminotransferase (ALT), emphasizing the bioclinical relevance of the assay. Pretreatment serum HCV RNA levels above 10^5 copies/ml were found more frequently in nonresponders than in responders (76% vs. 44%; $P < 0.05$). Given their great sensitivity and the significant correlations, the Amplicor™ HCV qualitative and quantitative assays appear useful for the diagnosis and management of hepatitis C infection, and especially for monitoring of therapy. ***J. Med. Virol. 52:105–112, 1997.***

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INTRODUCTION

Infection by hepatitis C virus (HCV) often leads to a chronic state. However, the rate of progression of liver disease, development of cirrhosis, and hepatocellular carcinoma are extremely variable. A great heterogeneity in the response rate to treatment with interferon, defined initially as a return to normal alanine aminotransferase (ALT) values, has also been found in different clinical trials [Davis et al., 1989; Di Bisceglie et al., 1989; Trepo et al., 1994]. Improvement of clinical features of the disease is associated with a decrease or loss of HCV from serum or liver. It has been suggested that response to therapy depends primarily on virological criteria such as viral load and HCV genotypes [Hino et al., 1994; Martinot-Peignoux et al., 1995; Orito et al., 1994; Shindo et al., 1995; Yamada et al., 1995]. Thus virological surrogate markers appear to be needed to follow patients with HCV infection.

The most sensitive way to detect HCV virus is the amplification of viral RNA by reverse transcription and a polymerase chain reaction (RT-PCR). To quantify HCV RNA, a signal amplification technology, the branched DNA (bDNA) assay (Quantiplex™, Chiron Diagnostics), has been developed [Lau et al., 1993a]. This is a standardized method which is highly reproducible. However, its sensitivity is at least 100-fold lower than that of RT-PCR.

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TABLE I. Main Characteristics of the Patients

Characteristics	n = 48
Age (years)	46.6 (25–70)
Sex (M/F)	35/13
Blood exposure	12 (25%)
Drug users	9 (18.7%)
CAH	41 (85%)
Active cirrhosis	7 (15%)

Attempts to measure HCV RNA levels by RT PCR have been done by semiquantitative tests [Bresters et al., 1994; Shindo et al., 1994; Whitby and Garson, 1995] or competitive tests [Clossais Besnard and Andre, 1994; Gretch et al., 1994; Kumar et al., 1994; Ravaggi et al., 1995] developed in individual laboratories. Analysis of these results emphasizes variabilities inherent to the PCR technology itself, but also the use of different experimental conditions, varying from one laboratory to the other. The use of a standardized RT PCR test by all the laboratories would be critical to improve the intralaboratory reproducibility and to compare the results of the efficiency of treatment from one laboratory to another. Standardized RT-PCR assays for detection (the Amplicor™ HCV assay) and quantification (the Amplicor HCV Monitor™ assay) have been developed (Roche Molecular Systems). These 2 tests use the same protocol, but the quantitative format includes an internal standard for the control of reaction efficiency and possible sample interference.

We evaluated these 2 assays in terms of specificity, sensitivity, and HCV RNA levels in comparison to the Quantiplex™ assay and an in-house RT PCR, retrospectively, in 48 patients with chronic HCV infection before, during, and after interferon treatment.

MATERIALS AND METHODS

Patients

Sera were obtained from 48 anti-HCV-positive patients with chronic liver disease: 41 with chronic active hepatitis (CAH) and 7 with active cirrhosis. Characteristics of the patients are shown in Table I. All the patients had serum aminotransferase levels above the normal limit for at least 6 months and were negative for serum HBsAg and human immunodeficiency virus antibody. Liver disease was confirmed by biopsy in all patients. Seven patients were liver transplant recipients and presented an HCV reinfection of the liver graft. These patients were treated with a combined interferon-ribavirin therapy [Bizzolon et al., 1996]. The other 41 patients received interferon therapy alone: 39 with recombinant alpha interferon, 6 with lymphoblastoid interferon, and 3 with recombinant beta interferon. The duration of therapy varied from 6 to 18 months and the dosage from 3 to 16 MU three times weekly. Interferon efficacy was assessed on the basis of serum ALT level and HCV RNA titer at the end of therapy and 6 months later.

Detection and Quantification of HCV RNA With the Amplicor™ HCV and Amplicor HCV Monitor™ Assays

The qualitative and quantitative Amplicor™ HCV assays were carried out according to the manufacturer's instructions. Briefly, HCV RNA from 100 μl of serum was extracted in a lysis buffer and then precipitated with isopropanol. The pellet was resuspended in 1 ml of sample diluent and 50 μl were reverse transcribed and amplified with the rTth DNA polymerase in the Perkin Elmer thermal cycler 9600. The single set conditions also include primers in the 5' noncoding (NC) region, the antisense primer biotinylated, and the use of the UNG enzyme to prevent contamination from previous PCR reactions. The PCR products were detected by an ELISA-based method. The amplified HCV cDNA was denatured and hybridized on wells of a plate coated with the specific probe, and the biotin labeling was detected by colorimetric reaction following the incubation with avidin peroxidase.

In the quantitative format (Amplicor HCV Monitor™) 100 copies of internal standard (quantification standard, QS) were added with serum sample at the time of extraction. After amplification and denaturation, serial fivefold dilutions of PCR products (1:1, 1:5, 1:25, 1:625) were added to wells of plate coated with a probe specific for the wild type cDNA and to other wells coated with the QS-specific probe. After the colorimetric reaction was carried out on the hybridized product, the titer was calculated from the absorbance of the highest dilutions of the PCR product having an OD in the range 0.2–2.0 with the wild type and the QS probes, respectively. The data entered in a spreadsheet of an Excel program provided by Roche Molecular Systems are processed automatically.

Measurement of HCV RNA Level by bDNA Assay Quantiplex™ HCV Version 1.0

This signal amplification assay is based on a sequence of hybridization steps. Duplicate 50 μl of serum were added to the wells of a microtiter plate in a lysis buffer containing a series of oligonucleotide probes complementary to the 5' NC and core regions of the HCV RNA, and mediating HCV RNA capture to the wells and binding of the bDNA molecule. After overnight incubation and washing, the subsequent steps included binding of the bDNA amplifier, then of the alkaline phosphatase-labeled probe, and chemiluminescence reaction with dioxetane substrate. Multiple copies of the labeled probe hybridize to each bDNA molecule, thereby amplifying the signal. Light emission is proportional to the target RNA level. HCV RNA concentration is determined from standard curve.

HCV RNA Detection by RT PCR

HCV RNA was extracted from 200 μl of serum with the guanidium thiocyanate phenol chloroform method and reverse transcribed into cDNA in the presence of antisens primer as previously described [Li et al.,

1995]. Half of the cDNA was amplified for 45 cycles (95°C for 45″, 64°C for 45″, 72°C for 1′), with the NC3 (5-CCTGTGAGGAACTACTGTCTTCACGCA-3) and NC4 (5-ACTCGCAAGCACCCTATCAGGCAGTAC-3) primers from the 5′NC region [Li et al., 1995], in a final volume of 50 μl. Fifteen μl of the PCR products were electrophoresed in a 2% agarose gel and analyzed by Southern blot hybridization with the NC-H probe (5-AGCCGAGTAGTGTTGGGTCGCG-3).

Determination of the HCV Genotype

Genotyping was performed with the InnoLipa HCV method (Innogenetics), according to the manufacturer's instructions.

RESULTS

Comparison of the "In-House" PCR and the Amplicor™ HCV Sensitivities

From the 85 samples tested by the qualitative Amplicor™ HCV and the in-house PCR, 70 (82%) were concordant. Fifty-four (63.5%) and 16 (19%) were positive and negative, respectively in the 2 assays. Only 1 was found positive in the in-house RT-PCR and negative by the Amplicor™ HCV assay. The 14 other discrepant samples (16%) were positive by the Amplicor™ HCV and negative by the in-house PCR. The Amplicor™ HCV assay appeared, therefore, to be more sensitive than in-house PCR.

Of the 65 samples positive with Amplicor™ HCV assay, tested by the in-house RT-PCR and the Amplicor HCV Monitor™ assay, 42 (72%) were found positive by the 2 latter assays, 5 (8%) were positive by the in-house RT-PCR but not detected by the Amplicor HCV Monitor™ assay, 8 (12%) were positive by the Amplicor HCV Monitor™ assay but negative by the "in house" RT-PCR, and 5 (8%) were negative by both assays. Thus a concordance of the qualitative results between the Amplicor™ HCV assay and the in-house RT-PCR was observed in 80% of the samples, and between the Amplicor™ HCV and the Amplicor HCV Monitor™ assays in 85% of the samples. For 20% (13/65) of the positive samples, the detection by the in-house RT-PCR and the Amplicor HCV Monitor™ assay differed. For these 13 sera, no evidence of the presence of an inhibitor has been found since the OD with the internal standard in the Amplicor HCV Monitor™ assay were as high as those obtained after coamplification with the other specimens, including the positive and negative controls of the kit run in the same experiment.

Quantitative Measurements with the Amplicor HCV Monitor™ Assay

For 83% of the samples tested, a value above cutoff has been obtained, with HCV RNA copy numbers varying from 324 to 3.91×10^6/ml. The cutoff has been calculated by assigning an OD of 0.20 to the samples which have given an OD below 0.20 for undiluted PCR product. The number of HCV RNA copies per ml in these samples was considered to be less than this calculated cutoff value. For 31 (17%) samples that could not be titrated, the calculated cutoff was below 1000 copies/ml in 20 and above 1000 copies/ml in 11 (but below 2000 copies/ml in 9/11).

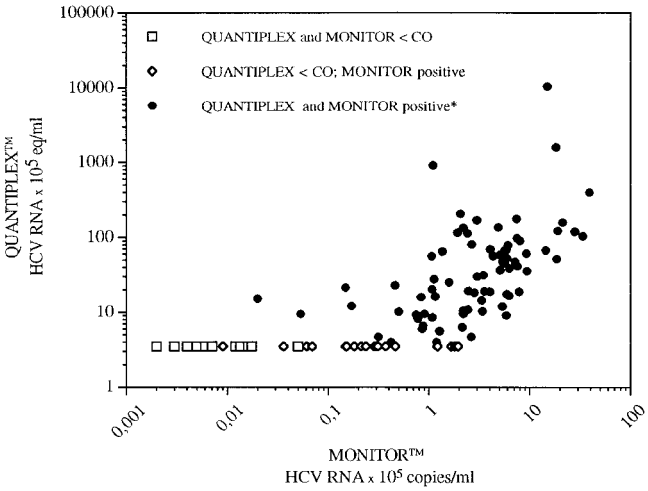

Fig. 1. Relationship between HCV RNA levels measured by Amplicor HCV Monitor™ and Quantiplex™ assays in 118 follow-up samples from 35 interferon-treated patients. *The best fit line of the linear regression analysis of the data from specimens with an HCV RNA level above Quantiplex™ cutoff (CO) is described by the equation $\log_{10}$ (Quantiplex) = 1.3 + 0.6 × $\log_{10}$ (Monitor).

HCV RNA levels greater than the cutoff with the Amplicor HCV Monitor™ assay were found below the detection limit of the Quantiplex™ assay in 22% (22/98) of the positive specimens, and 52% (22/42) of the samples with a Quantiplex™ value below 3.5×10^5 eq/ml were measurable by the Amplicor HCV Monitor™ assay, giving titers from 487 to about 200,000 copies/ml. For samples positive with the 2 assays, we found a significant correlation (Fig. 1) between the 2 respective $\log_{10}$ values ($r = 0.6$; $P < 0.0001$), despite higher titers found with Quantiplex™ assay compared to those found with the Amplicor HCV Monitor™ assay (median of the Quantiplex to Monitor values ratio = 9.6; one-sample sign test $P < 0.0001$).

Quantification of HCV RNA and Responses to Therapy

Results obtained from analysis of samples from treatment follow-up also showed a good correlation between the Amplicor HCV Monitor™ and Quantiplex™ assays. The time course of viremia with the 2 assays during and after treatment of 8 patients is shown in Figure 2.

Seven patients were liver transplant recipients, under a combined interferon-ribavirin therapy [Bizzolon et al., 1996]. These 7 patients clearly behaved as a separate group with a very high viral titer compared to the other patients, and at the end of combination therapies, a complete biochemical response was observed in almost all of them, regardless of the virological response (not shown). Thus these patients were excluded from the analysis of factors of response to therapy.

Table II shows for the 41 other patients the genotype

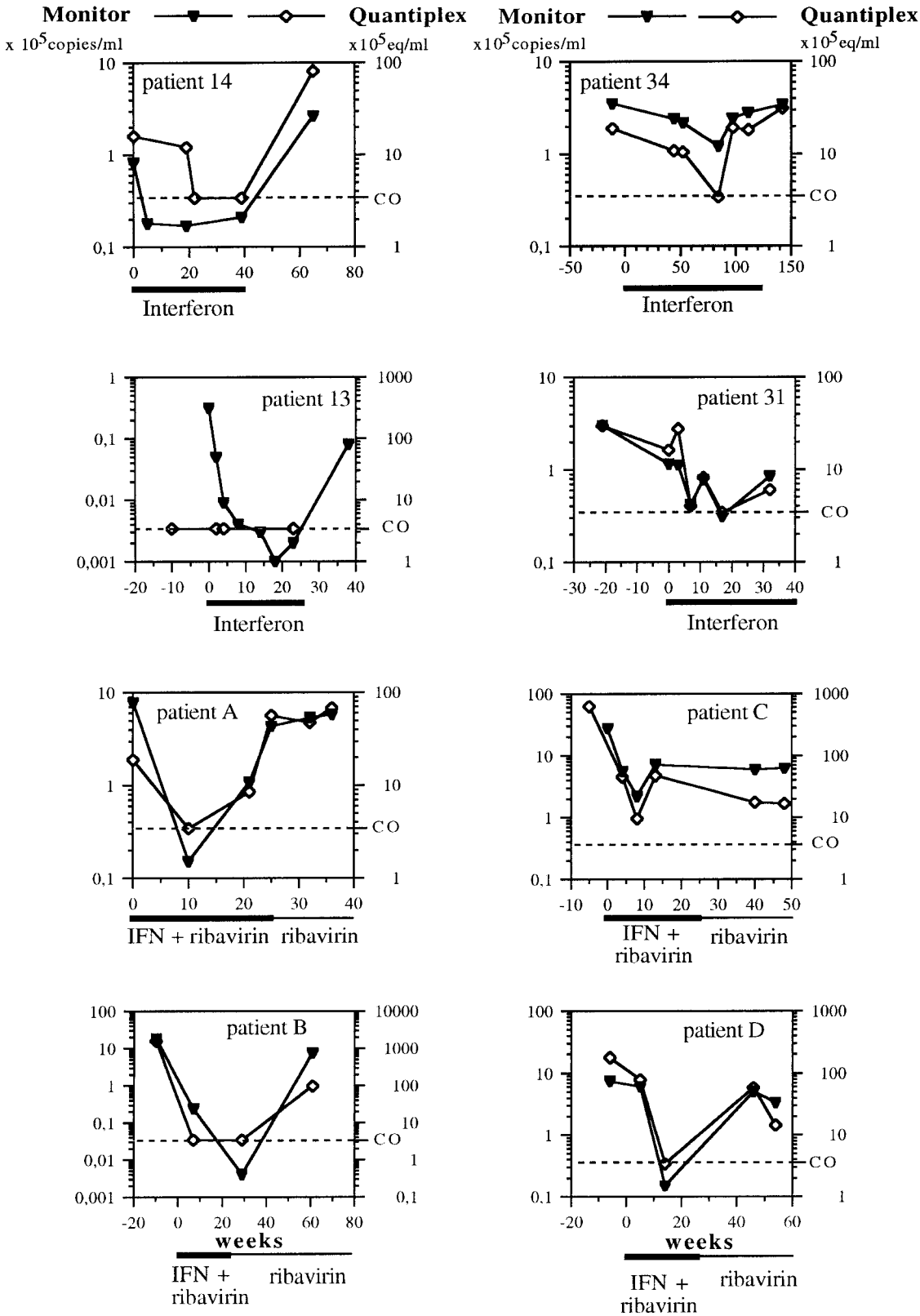

Fig. 2. Follow-up of HCV RNA levels during interferon or interferon plus ribavirin treatments of HCV infected patients, determined by the Amplicor HCV Monitor™ and the Quantiplex™ assays. Dashed lines indicate Quantiplex™ cutoff. Nature and duration of treatment are noted under the X axis. Patients A, B, C, and D are liver transplant recipients.

TABLE II. Baseline HCV RNA Level and Therapeutic Response in Interferon-Treated Patients

Patient	Genotype	Pretreatment HCV RNA level Monitor™ assay × 10^5 copies/ml	Interferon response[a]	
			Virological HCV RNA	Biochemical ALT
1	1a	<0.004	CR	CR
2	1a	0.46	CR	CR
3	1a	7.47	CR	CR
4	1a	9.27	CR	CR
5	1a	+[b]	CR	CR
6	1a	0.90	Rel	CR
7	1b	1.19	Rel	CR
8	3a	1.93	Rel	CR
9	1a	0.884	Rel	Rel
10	1a	1.40	Rel	Rel
11	1a	14.51	Rel	Rel
12	1b	0.31	Rel	NR
13	1a	0.83	NR	CR
14	1b	9.38	NR	CR
15	1a	0.004	NR	Rel
16	1a	0.017	NR	Rel
17	1a	0.053	NR	Rel
18	1b	0.038	NR	Rel
19	1b	0.26	NR	Rel
20	1b	5.32	NR	Rel
21	1a	0.005	NR	NR
22	1a	0.37	NR	NR
23	1a	1.25	NR	NR
24	1a	4.83	NR	NR
25	1a	7.9	NR	NR
26	1a	18.59	NR	NR
27	1a	39.07	NR	NR
28	1b	0.15	NR	NR
29	1b	0.43	NR	NR
30	1b	1.04	NR	NR
31	1b	1.15	NR	NR
32	1b	1.36	NR	NR
33	1b	2.71	NR	NR
34	1b	3.54	NR	NR
35	1b	4.04	NR	NR
36	1b	6.23	NR	NR
37	1b	7.54	NR	NR
38	1b	10.49	NR	NR
39	1b	11.81	NR	NR
40	1b	19.04	NR	NR
41	1b	+[b]	NR	NR

[a]CR, complete responder during and 6 months after treatment; Rel, relapser with response at the end of the treatment which is not sustained after stopping interferon; NR, nonresponder at the end of the treatment even if HCV RNA (or ALT) decreased or is transiently negative during interferon therapy.
[b]Samples tested with only the qualitative format of the Amplicor™ HCV RNA assay.

and HCV RNA copy number obtained with the Amplicor HCV Monitor™ assay before treatment and the response to treatment at the virological and biochemical levels. Twenty (49%) were infected with HCV of genotype 1a, 20 (49%) with HCV type 1b, and 1 with HCV type 3a.

Only 5 of the 41 patients (12%) were sustained virological and biochemical responders who cleared serum viral RNA for at least 6 months after the end of treatment. These 5 patients were infected with HCV genotype 1a. Of the 5 biochemical sustained responders who remained viremic, genotype 1a was found in 2, genotype 1b in 2, and genotype 3a in 1.

In 29 patients (71%) the biochemical and virological responses were completely concordant: 5 patients were complete responders, 3 were relapsers, and 21 were nonresponders. For 11 (27%), ALT normalization was associated with a detectable viremia during or after treatment, suggesting a persistence or relapse of viremia in spite of a favorable response at the biochemical level. In contrast, ALT did not return to normal level in 1 patient (subject 12) although viremia disappeared during interferon therapy. Viral replication, however, resumed after the end of treatment.

Discordances between the Amplicor HCV Monitor™ and the Quantiplex™ assays were detected in 25% of the patients who were not sustained virological responders; 10% showed a relapse with the Amplicor

HCV Monitor™ assay but a complete response with the Quantiplex™ assay, and 15% were Amplicor HCV Monitor™ nonresponders but were relapsers with the Quantiplex™ assay. In all but 2 of these discordant patients with the 2 HCV RNA quantitative tests, the results with the Quantiplex™ were in accordance with those from the transaminases. In 2 cases (subjects 13 and 14), ALT normalized despite persistence of viremia by Amplicor HCV Monitor™ assay and a relapse pattern by Quantiplex™ assay.

Among virological nonresponders, those with transaminase normalization but relapse after treatment have a lower baseline viral load than those without a biochemical response during treatment (0.95 vs. 7.24 × 10^5 mean RNA copies/ml; $P < 0.01$, Mann-Whitney test). However, in 4 of the 6 virological nonresponders who were biochemical relapsers, viremia increased during treatment with a + 0.6 to 1.5 log difference between the end of therapy and the beginning. In contrast, the RNA titer of the 2 virological nonresponders with a sustained ALT normalization (subjects 13 and 14) decreased, remaining 0.6 and 2 log, respectively, below baseline level at the end of treatment. In the concordant nonresponders the evolution of viremia was variable, from a more than 0.5 log increase in 4 to a more than 0.5 log decrease in 3, but in 63% of them a maintenance of RNA level in the +0.5 to −0.5 log range occurred. Thus a biochemical response associated with a low baseline viral load is likely to be followed by a rebound of the transaminases, after cessation of therapy, if an increase in HCV RNA level occurs during treatment, while a sustained response may be obtained if the viremia decreased significantly, even not completely, with interferon.

Attempts to correlate pretreatment viral loads with genotype or interferon response could be carried out only during treatment due to the very low number of sustained responders.

Among patients infected with HCV type 1 (a + b), biochemical nonresponders had, significantly more frequently, a pretreatment viremia higher than 10^5 copies/ml than did responders (76% versus 44%; $P < 0.05$, χ^2 test). A pretreatment viral load greater than 10^5 copies/ml tended to be seen more frequently in patients infected with HCV genotype 1b (74%) than in those infected with HCV type 1a (50%), but this difference did not reach significance.

DISCUSSION

We evaluated in interferon-treated patients the Amplicor™ HCV qualitative and Amplicor HCV Monitor™ quantitative HCV RNA assays, in comparison to the in-house RT-PCR assay for the detection of HCV RNA, and the Quantiplex™ assay for quantification of HCV RNA. These Amplicor™ HCV assays proved to be useful clinically, especially because of the increased sensitivity relative to the in-house PCR or Quantiplex™ (version 1.0).

The Amplicor HCV Monitor™ quantitative format is also less sensitive than the corresponding Amplicor™ HCV qualitative format. The overall sensitivity of the Amplicor HCV Monitor™ quantitative assay is similar to that of our in-house qualitative HCV RNA PCR assay (85% and 80% respectively, compared to the Amplicor™ HCV assay). Nevertheless, the samples giving discordant results between the Amplicor™ HCV and the Amplicor HCV Monitor™ assays, on one hand, and the Amplicor™ HCV and the in-house HCV RNA PCR assay, on the other hand, are not always the same. Thus, in addition to a strict difference in sensitivity, the specificity may also differ from one PCR protocol to another. This is probably related to the different experimental conditions used (primers and probes). The Amplicor™ HCV and Amplicor HCV Monitor™ assays are expected to have identical specificity, since they use the same procedure. Thus, the discrepancy between these 2 tests might reflect only a difference in sensitivity, while the discrepant results between these 2 tests and our in-house PCR might depend on a difference in sensitivity as well as in specificity.

As expected, and as predicted by their respective detection limit, we found that the Amplicor HCV Monitor™ assay detected more samples than the Quantiplex™ assay (81% vs. 62%). Indeed, the Amplicor HCV Monitor™ assay is able to detect very low levels of viremia. From our results, the sensitivity appeared to be 1000 copies/ml. For 65% of undetected specimens, the calculated cutoff was below 1000 copies/ml. The few samples unreactive but with a cutoff above 1000 copies/ml represented 7% of the specimens for which we could determine the titer.

A correlation between the Amplicor HCV Monitor™ and Quantiplex™ assays in terms of HCV RNA levels was observed. However, titers found with the Amplicor HCV Monitor™ assay were lower than those observed by the Quantiplex™ assay. This discordance remains to be explained. Differences in technical features or concentration of RNA standard might be involved. Recently, by comparing the Amplicor HCV Monitor™ assay with a competitive, quantitative in-house RT-PCR assay, Roth et al. [1996] also found lower HCV RNA levels with the Amplicor HCV Monitor™ assay for the samples with the higher HCV RNA titer (>5 × 10^5 copies/ml). Underestimations may result from a saturation phenomenon of the Amplicor HCV Monitor™ for high HCV RNA copy numbers.

Despite the difference in titers the Amplicor HCV Monitor™ assay appeared as valuable as the Quantiplex™ assay for monitoring interferon treatment, since the evolution of RNA titers was identical with the 2 assays. Furthermore, the Amplicor HCV Monitor™ assay can detect persistent viremia in specimens that fell below the Quantiplex™ detection limit. Thus with the Amplicor HCV Monitor™ assay a likely subsequent biochemical relapse can be predicted earlier than with the Quantiplex™ assay.

The marker most often used to evaluate the response to therapy in HCV infected patients is ALT level. A discrepancy between virological and biochemical responses to interferon has been reported in some pa-

tients [Lau et al., 1993b], often associated with a persistent viremia (or fluctuation of viremia) in patients with ALT normalization following initiation of the treatment. This discrepancy was also noted in 12 of over 41 patients (29%), during (22%) or after (7%) treatment. In most of these cases (92%), the virus was detected while ALT became normal. Nevertheless, in 1 patient, circulating HCV RNA became undetectable under interferon therapy, although he was a nonresponder at the biochemical level. In this case, a swift virological rebound after discontinuation of treatment confirmed that HCV replication was continuing.

The small size of each group did not allow for a comparison of the kinetics of decrease in viral titers between biochemical sustained responders and relapsers, but it should be noted that all but 2 patients who remained HCV RNA positive while normalizing their ALT during treatment relapsed after IFN discontinuation, and this occurred in subjects with a relatively low baseline HCV RNA level but which paradoxically increased despite interferon therapy.

Whether determination of viral load does represent a good prognostic marker for interferon response is a crucial issue. It has been shown that a Quantiplex™ HCV RNA titer below 10^6 (or 2×10^6) equivalents/ml is more frequently associated with a good response, whereas the response rate is lower among patients with a HCV RNA level above this value [Orito et al., 1994; Yamada et al., 1995]. We found that patients with more than 10^5 copies/ml of HCV RNA with the Amplicor HCV Monitor™ assay are more likely to be nonresponders than patients with RNA levels below this value. This is consistent with results obtained with the Quantiplex™ assay, taking into account a ten-fold difference in titers observed between the 2 assays.

Given the importance of measuring viremia in the monitoring of HCV therapy, one concern is to select the best performing assay to this end. If the objective is to test many specimens, easy, reproducible standardized tests are needed. The 2 quantitative commercial assays used are quite satisfactory. Our results showed that the Amplicor HCV Monitor™ and the Quantiplex™ assays displayed overall good concordance, including for evaluation of samples obtained from therapeutical protocols, although for individual follow-up the same assay should be used. The advantage of the Amplicor HCV Monitor™ assay is its higher sensitivity compared to that of version 1.0 of the Quantiplex™ assay. The recent development of a second generation Quantiplex™ assay with a cutoff of 200,000 instead of 350,000 equivalents/ml will undoubtedly reinforce the similarities between the 2 tests. Larger studies should be undertaken to confirm these observations and the usefulness of this assay for assessment of responses to treatment.

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